**TRANSLATION**

I, Yuko Mitsui, residing at 4-6-10, Higashikoigakubo, Kokubunji-shi, Tokyo, Japan, state:

that I know well both the Japanese and English languages, that I translated, from Japanese into English, Japanese Patent Application No. 2000-087500, filed on March 27, 2000, and that the attached English translation is a true and accurate translation to the best of my knowledge and belief.

Dated: January 20, 2005

Yuko Mitsui

Yuko Mitui

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: March 27, 2000

Application Number: Patent Application No. 2000-087500

Applicant(s): OLYMPUS OPTICAL CO., LTD.

This 27th day of September 2001

Commissioner,
Patent Office Kozo OIKAWA (seal)

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[Title of the Invention] METHOD FOR DETERMINING POLYMORPHISM GENE

[Number of Claims] 3

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[List of Items Submitted]

| | | |
|---|---|---|
| [Name of Item] | Specification | 1 |
| [Name of Item] | Drawings | 1 |
| [Name of Item] | Abstract | 1 |

[Number of General Power of Attorney] 9602409

[Necessity of Proof] Necessary

[Document] SPECIFICATION

[Title of the Invention] METHOD FOR DETERMINING POLYMORPHISM GENE

[What is claimed is:]

[Claim 1] A method of determining which of the polymorphism sequence $PS_1$ to $PS_n$ (n is an integer 2 or more) corresponds to the nucleotide sequence of the polymorphism site contained in the polynucleotide, comprising the steps of:

preparing a test sample containing a polynucleotide;

mixing a test sample with DNA probes $PR_1$ to $PR_n$ labeled with a detectable marker and capable of specifically binding to polymorphism sequences $PS_1$ to $PS_n$, thereby binding the DNA probes $PR_1$ to $PR_n$ to the polynucleotide;

detecting the DNA probes $PR_1$ to $PR_n$ present in a micro space; and

analyzing detection results to determine, which one of the DNA probes $PR_1$ to $PR_n$ binds to the polynucleotide, thereby determining which one of the polymorphism sequences $PS_1$ to $PS_n$ corresponds to a nucleotide sequence of the polymorphism site.

[Claim 2] The method according to claim 1, characterized in that the detecting is performed by a confocal microscope, and said analyzing is performed by a fluorescent correlation spectroscopy.

[Claim 3] The method according to claim 1, characterized in that the polynucleotide is a gene for a human histocompatible antigen.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to a method for determining polymorphism in a polynucleotide.

[0002]

[Prior Art]

In recent years, with the progress of Genome Science, the link between specific disorders and gene polymorphism has been rapidly elucidated. It is expected that the analysis of gene polymorphism will be extremely useful in the diagnosis of genetic diseases. In the case of humans, the homology of genome DNAs between individuals exceeds 99%. A minor difference exists in a nucleotide sequence. For this reason, analysis of the SNP (Single Nucleotide Polymorphism) has drawn attention.

[0003]

As a method for analyzing polymorphism of a gene, a serological method and a method using DNA are primarily known. The latter method is superior to the former method in that a well-trained technician is not required for determination, and in that the examination step may be automatically performed.

[0004]

As the method for using DNA, a wide variety of methods are known. Most of them are a combination of prior arts, such as PCR-amplification, hybridization between a labeled probe and an amplified product, detection of a labeled substance and the like.

[0005]

Although these methods are easier in operation than the serological method, they have the following problems:

(1) PCR-amplification, washing, and electrophoresis involve complicated operation and a long time is required for the analysis;

(2) in the case where a polymorphism analysis is performed by using a probe fixed onto a plate such as a micro-titer plate, when the rate of polymorphism to be analyzed is high, not only that enormous amounts of test samples and reagents must be prepared, but also that items simultaneously determined are limited in number;

(3) the detection cannot be performed with a sufficient accuracy since an increase of background due to a remaining marker substance and a non-specific reaction takes place; and

(4) examination cost is high.

[0006]

[Object of the Invention]

The present invention has been made to solve the above problems, and an object thereof is to provide a method for determining a great number of polymorphism quickly, and simply.

[0007]

[Means for Achieving the Objects]

To solve the above problems, according to the present invention, there is provided a method of determining which of the polymorphism sequence $PS_1$ to $PS_n$ (n is an integer 2 or more) corresponds to the nucleotide sequence of the polymorphism site contained in the polynucleotide, comprising

the steps of: preparing a test sample containing a polynucleotide; mixing a test sample with DNA probes $PR_1$ to $PR_n$ labeled with a detectable marker and capable of specifically binding to polymorphism sequences $PS_1$ to $PS_n$, thereby binding the DNA probes $PR_1$ to $PR_n$ to the polynucleotide; detecting the DNA probes $PR_1$ to $PR_n$ present in a micro space; and analyzing detection results to determine, which one of the DNA probes $PR_1$ to $PR_n$ binds to the polynucleotide, thereby determining which one of the polymorphism sequences $PS_1$ to $PS_n$ corresponds to a nucleotide sequence of the polymorphism site.

[0008]

[Embodiments of the Invention]

According to the present invention, there is provided a method of quickly and simply determining the nucleotide sequence of the polymorphism site contained in a predetermined polynucleotide, more typically, in a polymorphism gene.

[0009]

In this text, the term "polymorphism gene" refers either to an allele group containing a plurality of types of alleles occupying a single genetic locus or to individual alleles belonging to the allele group.

[0010]

The term "polymorphism site" used herein is a site whose nucleotide sequence differs between polymorphism genes. For example, if the nucleotide sequence of a polymorphism gene A1 is AAA TTT ***CCC*** GGG and the nucleotide sequence of a polymorphism gene A2 is AAA TTT ***AGT*** GGG, the site indicated

by italic font corresponds to the polymorphism site. If a single nucleotide differs in the polymorphism site, such a polymorphism is particularly designated as a "single nucleotide polymorphism".

[0011]

The term "polymorphism sequence" used herein refers to a nucleotide sequence contained in a polymorphism site (in the example shown above, the "polymorphism sequence" is the nucleotide sequence indicated by italic font).

[0012]

According to an aspect of the present invention, there is provided a method for determining a nucleotide sequence contained in a polymorphism site of an arbitrarily chosen polymorphism gene. In other words, there is provided a method of determining the genotype of a polymorphism gene.

[0013]

Hereinafter, polymorphism sequences known to be contained in a polymorphism site of a polymorphism gene to be detected (hereinafter, referred to as a "target polymorphism gene") are represented by $PS_1$ to $PS_n$ (n is an integer of 2 or more). In the aforementioned example, $PS_1$ is, for instance, CCC and $PS_2$ is AGT.

[0014]

If the method according to an embodiment of the present invention is used, the type of a polymorphism site can be quite quickly determined. Thus, according to an embodiment of the present invention, it is possible to detect a polymorphism gene containing a plurality of polymorphism sites and a polymorphism

gene having a plurality of types of polymorphism sites. Examples of polymorphism gene include, but not limited to major histocompatible antigens containing a human leukocyte antigen (hereinafter referred to as "HLA").

[0015]

According to an embodiment of the present invention, when the type of a polymorphism site is determined, a test sample containing either a target polymorphism gene is first prepared. According to an embodiment of the present invention, when such a method is applied to humans, the test sample may be, but not limited to a body fluid including blood, spinal fluid, and cerebrospinal fluid.

[0016]

According to an embodiment of the present invention, it is possible to detect polymorphism with a high sensitivity. Therefore, a conventional PCR amplification operation may be omitted. However, if a target polymorphism gene is present in a small amount, PCR amplification may be performed. In the case where a target polymorphism gene is amplified by PCR, it is necessary to select a primer pair which sandwich a polymorphism site to be determined.

[0017]

Subsequently, nucleic acid probes $PR_1$ to $PR_n$ (n is an integer of 2 or more) containing nucleotide sequences complementary to respective polymorphism sequences $PS_1$ to $PS_n$ are mixed with the test sample. Since nucleic acid probe $PR_1$ contains a nucleotide sequence complementary to polymorphism sequence $PS_1$, only nucleic acid probe $PR_1$ can be bound to

a target polymorphism gene when the type of a target polymorphism gene is $PS_1$. Similarly, when target polymorphism genes are $PS_2$ to $PS_n$, they can bind to nucleic acid probes $PR_2$ to $PR_n$, respectively.

[0018]

As long as an unknown polymorphism sequence is not present in the target polymorphism gene, any one of polymorphism sequences $PS_1$ to $PS_n$ is included in the polymorphism gene. Therefore, if the polymorphism gene is mixed with nucleic acid probes $PR_1$ to $PR_n$, any one of nucleic acid probes $PR_1$ to $PR_n$ comes to bind to a polymorphism site of the polymorphism gene.

[0019]

Since each of the nucleotide probes $PR_1$ to $PR_n$ is labeled with a detectable marker substance, more preferably, with a fluorescent substance or luminescent substance, the polymorphism gene having the nucleic acid probe bound thereto can be detected by the detection operation described below.

[0020]

The marker substances to be attached to nucleic acid probes may be the same in type. However, it is preferable that at least two types of marker substances be used so as to determine which nucleic acid probe binds to the target polymorphism gene. When a single type of marker substance is used alone, it is possible to determine which nucleic acid probe binds to the target polymorphism target gene by changing the length of each of nucleic acid probes.

[0021]

After any one of nucleic acid probes $PR_1$ to $PR_n$ is bound to the target polymorphism gene, nucleic acid probes $PR_1$ to $PR_n$ present in "a micro space" are determined.

[0022]

The term "micro-space" be used in accordance with an embodiment of the present invention may have a volume of $10^{-21}$L (corresponding to the volume of a cube of 100 nm each side) to $10^{-3}$L. The micro-space may typically have a volume of $10^{-18}$L to $10^{-9}$L, and most typically, $10^{-15}$L to $10^{-12}$L. The micro-space may take any shape including a sphere, cylinder, cone, cube, or rectangular parallelepiped.

[0023]

The target polymorphism gene present in the micro space can be detected by detecting the marker of the nucleic acid probe bound to the gene. As describe above, since the volume of the micro space is extremely small, the detection is preferably performed by using laser light. More specifically, the fluorescence in the micro space is detected under microscopic field of view.

[0024]

The aforementioned detection can be performed by a confocal microscope as shown in FIG. 1. The confocal microscope itself has been known in the art.

The detection can be performed by use of the confocal microscope, comprising

(1) applying laser light as excitation light;

(2) passing laser light through a filter (IF), converging

and applying it to a single point of a sample by use of a dichroic mirror (DM);

(3) exciting a fluorescent substance in the sample by the laser light to emit fluorescence;

(4) passing the fluorescence through a pin hole to amplify fluorescence only emitted from the fluorescent substance within the focus of the sample by a multiplier phototube (PMT); and

(5) detecting the fluorescence thus amplified.

[0025]

FIG. 1 shows an example of an argon ion laser. Depending upon a type of a fluorescent substance, a krypton-argon ion laser, helium-neon laser, helium-cadmium laser different in wavelength may be used. FIG. 1 is only a schematic view of a typical confocal microscope. It is needless to say that a system other than the confocal microscope shown in FIG. 1 may be used.

[0026]

In the detection method, only fluorescence emitted from a single and micro point is detected. Therefore, the detection can be carried out without background and the detection sensitivity is significantly high compared to the fluorescent detection usually performed.

[0027]

The detection of a target polymorphism gene is performed for a time-period generally in the order of milli-second to minute, and most generally, in the order of second.

[0028]

After the detection results are obtained, the results are analyzed to determine which nucleic acid probe binds to the target polymorphism gene. If the type of nucleic acid probe bound to the target polymorphism gene is determined, the type of the target polymorphism gene can be elucidated.

[0029]

It is preferable that the detection results be analyzed by a fluorescent correlation spectroscopy. The fluorescent correlation spectroscopy (hereinafter referred to as FCS) is a technique of measuring fluorescent intensity of the average number of (or one, in some case) fluorescent substances for a predetermined time period, obtaining autocorrelation function of the fluctuation of fluorescence derived from Brownian movement, and analyzing the function to thereby acquiring various data items related to the fluorescent substances. FCS itself is known well. The FCS is described in detail in Patent Application No. 32017.

[0030]

In the present invention, FCS may be used in order to find which nucleic acid probe is bound to the target allele in the manner mentioned below.

[0031]

(1) Laser light is applied to the micro space shown in FIG. 2 (a).

[0032]

(2) The intensity of fluorescence emitted from a fluorescent substance present in the micro space is measured

with time to obtain data shown in FIGS. 2 (b) and 2 (c).

[0033]

(3) Calculating an expected value of a product $I(t) \times I(t+\tau)$ which are fluorescent intensities of two different time points, to obtain an auto correlation function:

Equation $G(\tau) = \langle I(t)I(t+\tau) \rangle$

[0034]

(4) The autocorrelation function obtained in the step (3) is analyzed by using the following equation 1:

[Equation 1]

$$G(\tau) = 1 + \frac{1}{N}\left[\left\{\frac{1-y}{1+\frac{\tau}{\tau_{free}}}\sqrt{\frac{1}{1+S^2\cdot\frac{\tau}{\tau_{free}}}}\right\} + \left\{\frac{y}{1+\frac{\tau}{\tau_{poly}}}\sqrt{\frac{1}{1+S^2\cdot\frac{\tau}{\tau_{poly}}}}\right\}\right]$$

[0035]

(where N is an average number of fluorescent molecules;

$\tau_{free} = wo^{2}/4D_{free}$: translational diffusion time of a free nucleic acid probe;

$\tau_{bound} = wo^{2}/4D_{bound}$: translational diffusion time of a nucleic acid probe bound;

y is a ratio of the nucleic acid probe bound; and

S is wo/zo

(wo is a diameter of a detection region; 2zo is a length

of a region, $D_{free}$ and $D_{bound}$ are translational diffusion constants of a nucleic acid probe unbound and a nucleic acid probe bound))

[0036]

(5) Autocorrelations before and after each of the nucleic acid probes is added.

[0037]

The FCS data analysis may be performed by a computer program "FCS" manufactured by Evotec BioSystem Co., Ltd.

[0038]

The concept of the analysis will be understood if referred to FIGS. 2 (b) and 2 (c). More specifically, when the nucleic acid probe is not bound to the target allele, the size of the molecule is small. As a result, the speed of the Brownian movement is high and the frequency is large in the function I(t). In contrast, when the nucleic acid probe is bound to the target allele, data of a large frequency can be obtained as shown in FIG. 2 (b). Hence, if analysis is made on the autocorrelation function obtained on the basis of the fluorescence intensity as mentioned above, whether the probe is bound or not can be elucidated.

[0039]

To determine which nucleic acid probe is bound by FCS, for example, each of the nucleic acid probes may be distinguishably labeled with fluorescent markers different in excitation wavelength and/or fluorescent wavelength.

[0040]

Furthermore, if the nucleic acid probes differs in size,

the Brownian movement and the autocorrelation function differ. Therefore, it is possible to determine which nucleic acid probe is bound to a sequence by using the nucleic acid probes different in size. It is needless to say that the type of nucleic acid probe bound to a target allele may be determined by using the probes different in size and a fluorescent marker.

[0041]

The target allele may be determined not only by a detection method using a hybridization reaction as an index but also by performing a PCR reaction using a labeled sequence which has been used as a probe, as a primer, and determining the type of marker of an amplified product. Furthermore, the PCR reaction may be used as an index. When such a PCR reaction is used as an index, if primers are designed so as to produce amplified products of different sizes depending upon types of polymorphism, the types of target allele can be determined based on the difference of autocorrelation functions ascribed to the different sizes of the amplified products.

[0042]

As described in the foregoing, if the present invention is employed, a type of polymorphism site of a target allele can be very quickly and simply determined.

[0043]

A method for determining the type of HLA will next be described below as an example of the present invention.

[Example 1] (see FIG. 3)

In this example, the type of polymorphism sequence showing an allo antigenicity to region DRB1 of HLA class II is

determined. DRB1*15 and DRB1*16 are subtypes of DR2. DRB1*15 (Sequence Number 1) and DRB1*16 (Sequence Number 2) differ only in 141st nucleotide (T→C) and 180th nucleotide (G→C).

[0044]

Probe 1, which has a nucleotide sequence complementary to the nucleotide sequence from the 141st to 180th nucleotide of DRB1*15, and probe 2, which has a nucleotide sequence complementary to the nucleotide sequence from the 141st to 180th nucleotide of DRB1*16, are prepared. Probe 1 is labeled with fluorescein isothiocyanate (FITC). Probe 2 is labeled with rhodamine. Each of probes 1 and 2 is added to a solution up to a concentration of about $10^{-8}$M.

[0045]

The nucleotide sequence of 60-200th nucleotides is amplified by PCR using a primer pair capable of specifically binding to a consensus region. Further, a solution is added up to a concentration of about 10-8 to prepare a DNA test sample.

[0046]

A drop of the DNA test sample is placed on a surface of a cover glass. After probe 1 and probe 2 are added, a hybridization reaction is performed at a temperature of about 58 to 60℃.

[0047]

A change in autocorrelation function of fluorescent luminescence is determined before and after the hybridization reaction.

[0048]

For example, in the case where the type of test sample

DNA is a DRB1*15 homotype, only probe 1 is bound. As a result, only the autocorrelation function of the yellow-green fluorescence emitted from FITC changes. On the other hand, in the case where the type of test sample DNA is a DRB1*16 homotype, only probe 2 is bound. As a result, only the autocorrelation function of the yellow-green fluorescence emitted from rhodamine changes. Furthermore, when the type of test sample DNA is a hetero of DRB1*15 and DRB1*16, probe 1 and probe 2 are bound. As a result, both autocorrelation functions of the fluorescence emitted from FITC and emitted from rhodamine change. If the type of test sample DNA is neither DRB1*15 nor DRB1*16, the autocorelation functions do not change at all.

[0049]

Probe 1 and probe 2 are labeled with different marker substances as described above. Therefore, if they are added in the same vessel, it is possible to determine which probe binds to a test sample DNA. In view of the recent finding that numerous polymorphism portions are present in HLA, the method of the present invention has a great advantage because analysis can be made by adding many types of probes simultaneously to the same vessel.

[0050]

In the method of this example, probes labeled with different marker substances are used. However, probes different in length may be used. In this case, each of probes must be selected so as to have the length which is large enough to determine the difference in fluctuation by FCS. For example,

in the aforementioned examples in which DRB1*15 and DRB1*16 are separately recognized, probe 1 and probe 3 (Sequence number 3; longer than probe 1 by 20 nucleotides) may be used.

[0051]

FIG. 4 shows an application example of the method of the present invention to a clinical test. According to the application example, the sub-class types of many polymorphism sequences can be quickly determined. Therefore, this method is usefully used to determine the type of an HLA since numerous polymorphism sites are present in the HLA and many subclasses are identified for each of the polymorphism sites.

[0052]

As is apparent from FIG. 4, polymorphism sequences (A2, A26, B40 ……) of an HLA are added to wells formed in a flat glass plate. More specifically, different polymorphism sequences are added to the wells of different columns. Thereafter, a probe group is added which is capable of binding specifically to subclasses of polymorphism sequences. Subsequently, which probe is bound is determined separately per well, as detailed in this example. In this manner, the subtypes of many polymorphism sequences can be determined. It is needless to say that that the polymorphism sequences to be added to the well are not limited to those of the HLA. Different polymorphism genes in type may be added to the wells.

[0053]

[Advantages of the Invention]

According to the method of the present invention, polymorphism sites of many types of polynucleotides can be

quickly and easily determined.

[Sequence Listing]

<110> Olympus Optical Co., Ltd.

<120> Method for determining a polymorphic sequence

<130>

<140>

<141>

<160> 3

<170> PatenIn Ver. 2.0

<210> 1

<211> 42

<212> DNA

<213> Homo sapiens

<400> 1

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<210> 2

<211> 42

<212> DNA

<213> Homo sapiens

<400> 2

ggaaacccca cctccgaggg tcctcctccg ccgcgcccga cg 42

<210> 3

<211> 59

<212> DNA

<213> Homo sapiens

<400> 3

tcgtctctca cagaaacccc acctccgagg gtcctcctcc gccgcgcccg cgccacga 59

[Brief Description of the Drawings]

[FIG. 1]

A scheme showing an example of a detection system which can be used in a method according to the present invention.

[FIG. 2]

Views showing the outline of a method for determining a polymorphism sequence by a detection method according to an embodiment of the present invention.

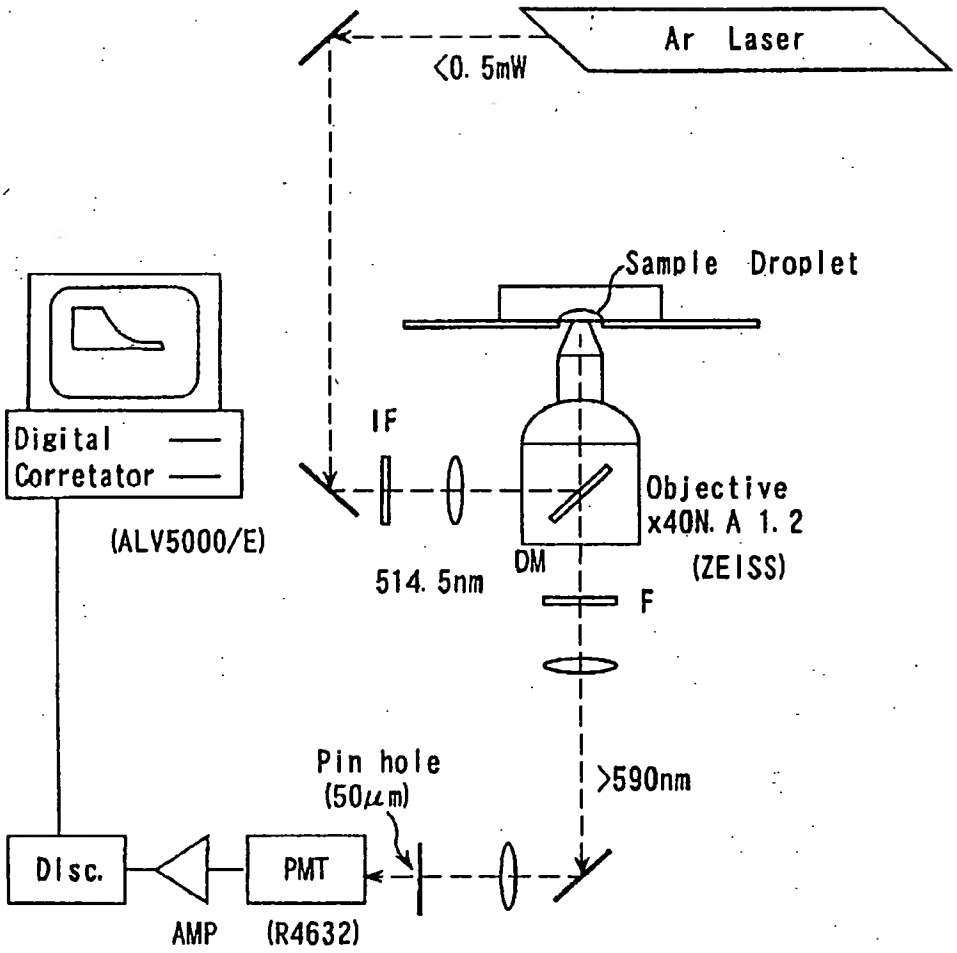
[FIG. 3]

A probe to be used in Example 1 of the present invention.

[FIG. 4]

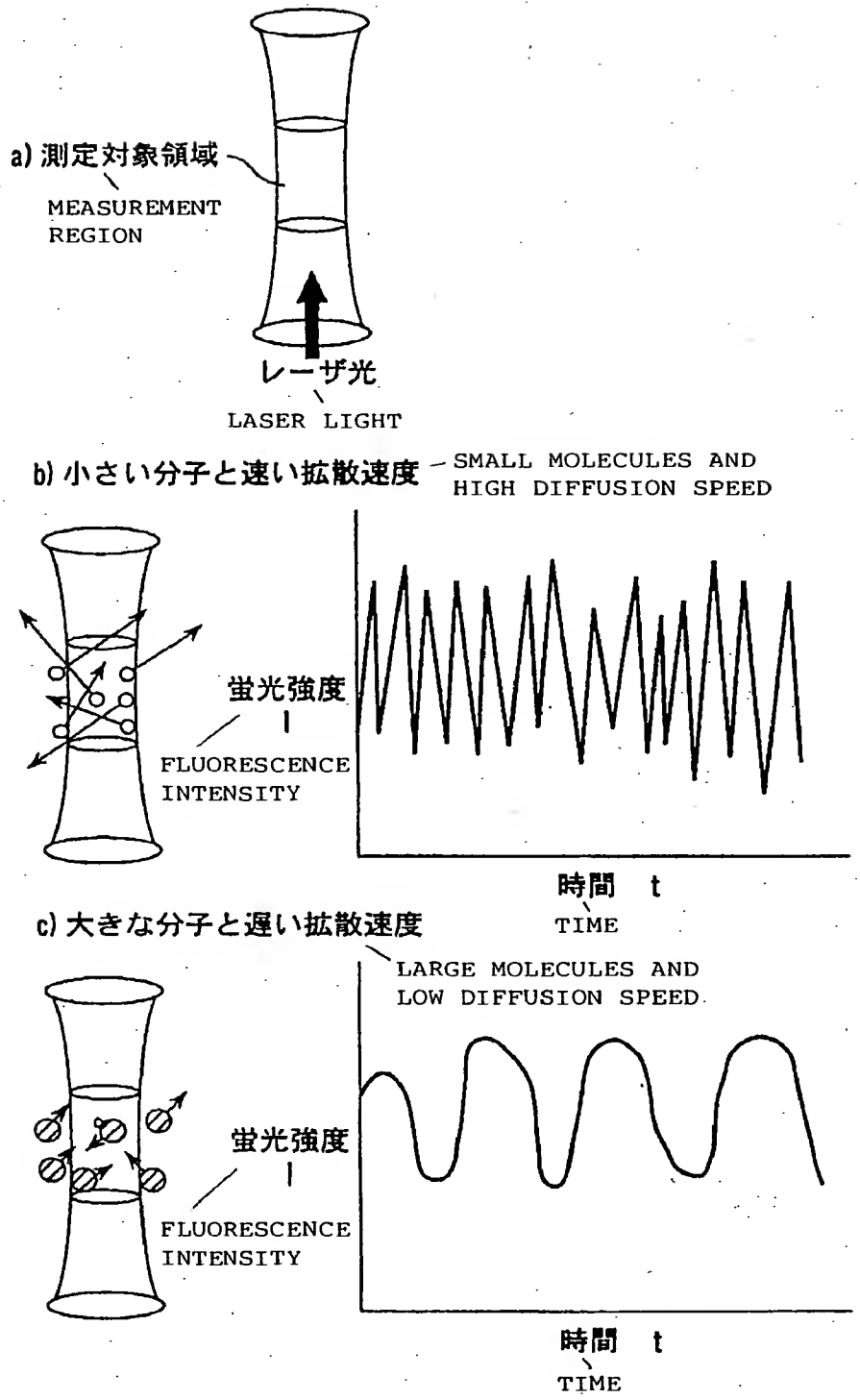
An application example of a method of the present invention in clinical medicine.

【書類名】　図面
[NAME OF DOCUMENT]　DRAWINGS
【図１】
[FIG. 1]



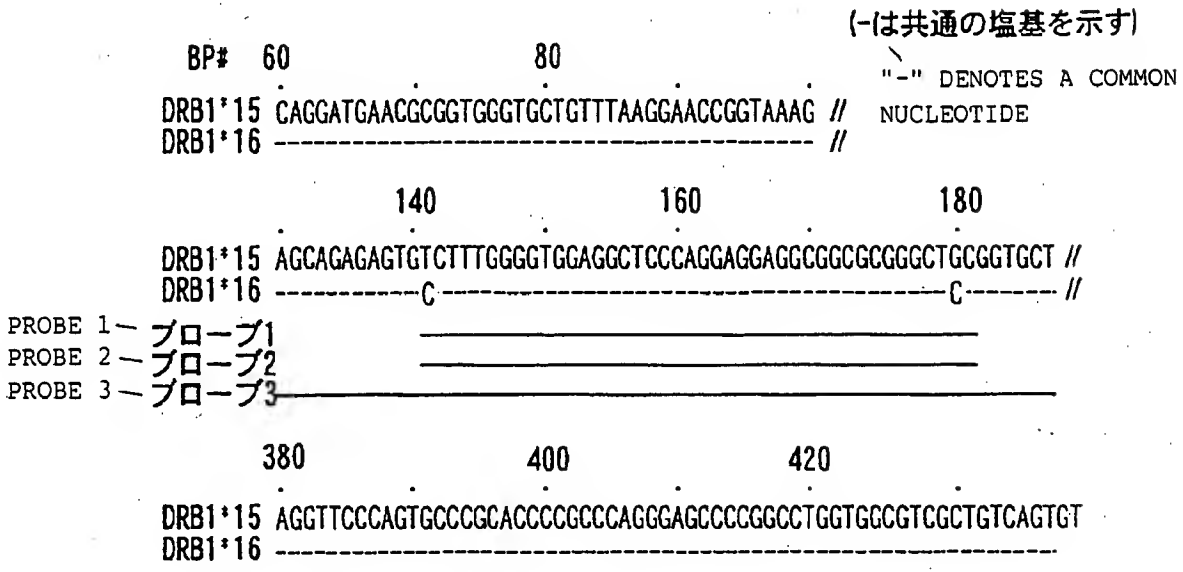
【図２】

[FIG. 2]


a) 測定対象領域
MEASUREMENT REGION
レーザ光
LASER LIGHT
b) 小さい分子と速い拡散速度
SMALL MOLECULES AND HIGH DIFFUSION SPEED
蛍光強度
FLUORESCENCE INTENSITY
時間 t
TIME
c) 大きな分子と遅い拡散速度
LARGE MOLECULES AND LOW DIFFUSION SPEED
蛍光強度
FLUORESCENCE INTENSITY
時間 t
TIME

【図３】

[FIG. 3]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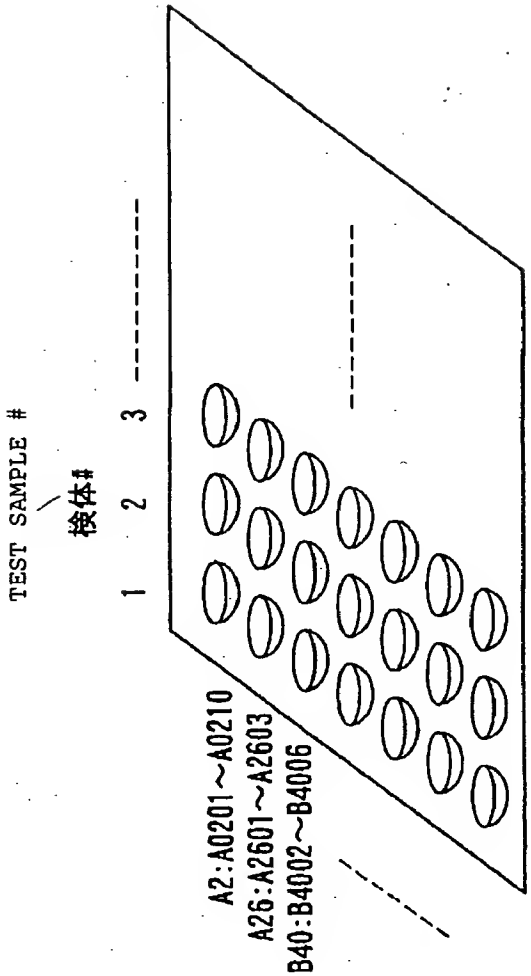
DRB1*16 ---------- //
140 160 180
DRB1*15 AGCAGAGAGTGTCTTTGGGGTGGAGGCTCCCAGGAGGAGGCGGCGCGGGCTGCGGTGCT //
DRB1*16 ----------C----------C------- //
PROBE 1 — プローブ1
PROBE 2 — プローブ2
PROBE 3 — プローブ3
380 400 420
DRB1*15 AGGTTCCCAGTGCCCGCACCCCGCCCAGGGAGCCCCGGCCTGGTGGCGTCGCTGTCAGTGT
DRB1*16 ----------

【図４】

[FIG. 4]


TEST SAMPLE #
検体#
1
2
3
A2:A0201～A0210
A26:A2601～A2603
B40:B4002～B4006

[Document] ABSTRACT

[Abstract]

[Objects] An object of the present invention is to provide a method for determining a nucleotide sequence of the polymorphism site contained in the polynucleotide quickly and easily.

[Means for Achieving the Objects] To solve the above problems, the present invention provides a method of determining the type of a polymorphism site contained in the polynucleotide. The method comprises the steps of: mixing the polynucleotide with one or more probes capable of binding to polymorphism sequences contained in the polynucleotide; detecting the polynucleotide present in a micro space using a confocal microscope; and analyzing detection results to determine the type of probe binding to the polynucleotide, thereby determining the type of the polymorphism site.

[Elected Figure] NONE

APPLICANT'S PAST DATA

Identification Number [000000376]

1. Date of Change August 20, 1990

[Reason for Change] New Registration

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